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INTERNAL STANDARDS FOR QUANTITATIVE GAS CHROMATOGRAPHY OF INDIVIDUAL BILE ACIDS AFTER GROUP SEPARATION OF BILE ACIDS IN URINE

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SUMMARY

Sodium glyco-7α,12α-dihydroxy-5β-cholan-24-oate, sodium tauro-7β,12β-dihydroxy-5β-cholan-24-oate and disodium glyco-7α,12α-dihydroxy-5β-cholan-24-oate 7α-sulphate have been synthesized for the first time. These compounds, together with 7β,12β-dihydroxy-5β-cholan-24-oic acid, which were added to a sample prior to extraction, were found to be useful as internal standards for determination by gas chromatography of individual bile acids in each fraction after group separation of urinary bile acids.

INTRODUCTION

Determination of bile acids by gas chromatography (GC) has been carried out by a number of complicated procedures. Most often, it is impossible to obtain 100% recovery of the bile acids in the sample. The usual method to correct for the loss of bile acids during the analysis is based either on adding an internal standard to the sample in the course of the systematic procedure or a recovery test using labelled and non-labelled bile acids under conditions similar to the analytical conditions. However, it cannot be ensured that recovery from a biological sample will be the same as that from a pure sample that has been prepared with labelled or non-labelled bile acids. Moreover, recovery may differ from assay to assay. Therefore, the addition of an internal standard to a sample before extraction is preferable.

Most of the bile acids in biological samples are composed of unconjugated (free), glycine- and taurine-conjugated and sulphated bile acids. In quantitative analysis of these bile acids, various compounds [1–11] that are usually used as internal standards are found only in the free bile acid fraction or neutral substance frac-

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tion. There have been no reports of suitable internal standards that can be added to the sample before extraction and also are separately transferred into all fractions when urinary bile acids are fractionated on the basis of the conjugation type before solvolysis and hydrolysis.

This paper describes the synthesis of internal standard compounds that are easily transferred into each conjugated bile acid fraction after group separation, and the application of these compounds to GC analysis of urinary bile acids.

EXPERIMENTAL

Materials

7α,12α-Dihydroxy-5β-cholan-24-oic acid ($\alpha\alpha$) and 7β,12β-dihydroxy-5β-cholan-24-oic acid ($\beta\beta$) were synthesized from cholic acid according to the method of Arimoto et al. [10]. Ethylglycinate hydrochloride, N,N-dimethylformamide (DMF) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) were purchased from Nakarai Chemicals (Kyoto, Japan). Dimethylethylsilylimidazole was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Other chemicals were of analytical-reagent grade, and all organic solvents were distilled before use.

The piperidinohydroxypropyl dextran (PHP) gel column was prepared by using PHP gel (Shimadzu, Kyoto, Japan). Amberlite XAD-2 resin was purchased from Rohm and Haas (Philadelphia, PA, U.S.A.).

Synthesis of internal standards

Glyco-7α,12α-dihydroxy-5β-cholan-24-oic acid, tauro-7β, 12β-dihydroxy-5β-cholan-24-oic acid and glyco-7α, 12α-dihydroxy-5β-cholan-24-oic acid 7α-sulphate were synthesized precisely according to the methods of Tserng et al. [12] for conjugation and Goto et al. [13] for sulphation. Melting points were determined on a hot-plate apparatus and were not corrected.

Sodium glyco-7α,12α-dihydroxy-5β-cholan-24-oate (glyco-$\alpha\alpha$-Na). A suspension of ethylglycinate hydrochloride (0.49 g) in 35 ml of ethyl acetate containing 0.5 ml of triethylamine was stirred at 25 °C for 0.5 h. EEDQ (0.78 g) and $\alpha\alpha$ (0.5 g) were then added to the solution. After stirring at 25 °C for 10 min, the suspension was refluxed overnight. The resulting suspension was cooled to room temperature and extracted with 30 ml of ethyl acetate. The ethyl acetate layer was separated, washed successively with 0.5 *M* sodium hydroxide, water, 0.5 *M* hydrochloric acid and water, and evaporated to dryness. The residue was dissolved in 10 ml of boiling ethanol, while 10 ml of 10% potassium carbonate solution were added slowly to the ethanolic solution to maintain a clear solution. The solution was heated for 15 min and then evaporated in vacuo to half its original volume. The solution was diluted with 20 ml of water, then acidified with 0.5 *M* hydrochloric acid. The precipitate was collected and dissolved in 0.5 *M* sodium hydroxide. The solution was percolated through a column of Amberlite XAD-2 resin. After washing with water, glyco-$\alpha\alpha$-Na was eluted with methanol. Recrystallization of the elute from methanol–water gave glyco-$\alpha\alpha$-Na (0.38 g) as colourless crystals, m.p. 257–262 °C.

Sodium tauro-7β,12β-dihydroxy-5β-cholan-24-oate (tauro-ββ-Na). Taurine (173 mg) and triethylamine (0.23 ml) were added to a stirred solution of ββ (0.5 g) and EEDQ (0.43 g) in 2.5 ml of DMF, and the resulting suspension was heated at 90°C until a clear solution was formed. The solution was stirred at 90°C for 15 min longer and then cooled to room temperature with stirring. The solution was poured slowly into 100 ml of stirred, chilled, anhydrous diethyl ether in an ice-bath. The resinous product was kept at 5°C overnight, and the supernatant was decanted. The resinous substance was washed several times with diethyl ether, dissolved in dichloromethane and filtered. The filtrate was evaporated to dryness. The residue was immediately dissolved in 25 ml of 0.2 *M* methanolic sodium hydroxide. The solution was diluted with 50 ml of anhydrous diethyl ether, kept at 5°C for several hours and then filtered. The collected solid was dissolved in water. The solution was adjusted to pH 10 with 0.5 *M* sodium hydroxide and percolated through a column of Amberlite XAD-2 resin. After washing with water, tauro-ββ-Na was eluted with methanol. Recrystallization of the elute from ethyl acetate–hexane gave tauro-ββ-Na (0.36 g) as colourless crystals, m.p. 198–200°C.

Disodium glyco-7α,12α-dihydroxy-5β-cholan-24-oate 7α-sulphate (glyco-αα 7-sulphate-Na). 7-Oxo-12α-hydroxy-5β-cholan-24-oic acid (m.p. 171–173°C, 2.3 g) [10] prepared from αα was acetylated in the usual manner [14]. 7-Oxo-12α-acetoxy-5β-cholan-24-oic acid (m.p. 245–248°C, 1.85 g) was dissolved in methanol and reduced with 2 g of sodium borohydride in an ice-bath overnight. The reaction mixture was poured into water and then acidified with hydrochloric acid to obtain 7α-hydroxy-12α-acetoxy-5β-cholan-24-oic acid (1.7 g, m.p. 205–207°C).

The above acid (0.5 g) and EEDQ (0.78 g) were then added to a suspension, which was prepared with ethylglycinate hydrochloride, ethyl acetate and triethylamine as described for glyco-αα-Na. The solution was stirred at 25°C for 10 min, followed by refluxing overnight. After cooling, the ethyl acetate layer was separated and washed successively with 0.5 *M* sodium hydroxide, water, 0.5 *M* hydrochloric acid and water. Then it was evaporated to dryness, and the residue was dissolved in 5 ml of anhydrous pyridine. To the ice-cooled solution was added chlorosulphonic acid in 5 ml of pyridine, and the solution was then heated at 50°C for 60 min. The resulting solution was poured into ice-water, acidified with concentrated hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with water, dried over sodium sulphate and evaporated to dryness. The crude product was dissolved in 10 ml of water adjusted to pH 10 with 0.5 *M* sodium hydroxide, and percolated through a column of Amberlite XAD-2 resin. After washing with water, glyco-αα 7-sulphate-Na was eluted with methanol. Recrystallization of the eluate from methanol–water gave glyco-αα 7-sulphate-Na (0.2 g) as colourless crystals, m.p. 181–184°C.

Methods

An outline of systematic procedure for analysis of urinary bile acids is given in Fig. 1.

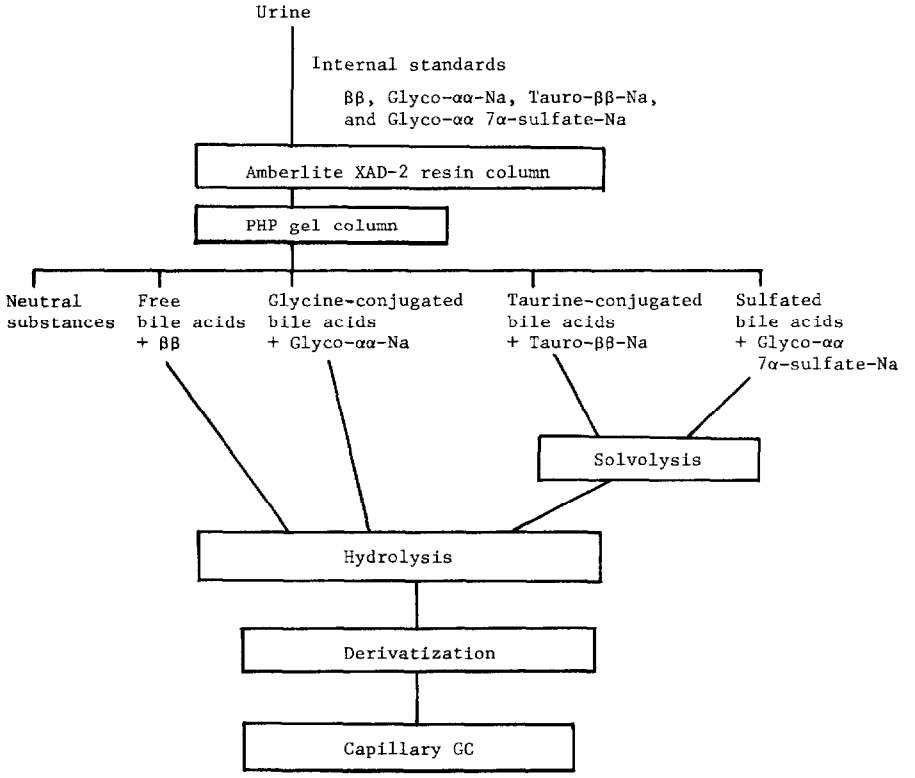

Fig. 1. Outline of procedure for systematic analysis of urinary bile acids.

Extraction of bile acids

For the analysis of urinary bile acids, a 10-ml aliquot of urine was mixed with four internal standards: 20 μg of $\beta\beta$, 25 μg of glyco-$\alpha\alpha$-Na, 25 μg of tauro-$\beta\beta$-Na and 30 μg of glyco-$\alpha\alpha$ 7-sulphate-Na, and then acidified to pH 4 with dilute hydrochloric acid. Urinary bile acids were adsorbed onto an Amberlite XAD-2 resin column (36×1 cm I.D.) The column was washed with distilled water till the effluent became neutral, and the bile acids were eluted from the column with 50 ml of ethanol containing 0.5 ml of 26% ammonium hydroxide. The effluent was evaporated to dryness. To separate the conjugated urinary bile acids, the residue was dissolved in three 0.5-ml volumes of 90% ethanol and placed on the top of the PHP gel column (3×0.4 cm I.D.). After removal of neutral substances with 6 ml of 90% ethanol, successive elution with 12 ml of 0.2 *M* acetic acid–90% ethanol, 12 ml of 0.3 *M* formic acid–90% ethanol, 16 ml of 0.3 *M* potassium acetate–90% ethanol and 16 ml of 1% ammonium carbonate–70% ethanol gave an unconjugated, a glycine-conjugated, a taurine-conjugated, and a sulphated bile acid fraction, respectively [15]. All fractions were evaporated to dryness.

Solvolysis and hydrolysis of bile acids

The unconjugated bile acids were hydrolysed in 3 ml of 2 *M* sodium hydroxide at 140°C for 4 h, because it was assumed that a part of conjugated bile acids might

elute into unconjugated bile acid fraction. The hydrolysate was adjusted to pH 1 with hydrochloric acid, and the free bile acids were extracted with diethyl ether.

The glycine-conjugated bile acids were hydrolysed, and the free bile acids were extracted as described for unconjugated bile acid fraction.

The taurine-conjugated bile acids were solvolysed on the assumption that sulphated bile acids might be eluted in the tauro-conjugated bile acid fraction. The tauro-conjugated bile acids were dissolved in 3 ml of ethanol. To the solution were added 27 ml of acetone–methanol (9:1, v/v) containing nine drops of 6 *M* hydrochloric acid. The mixture was solvolysed at 37°C for 16 h. The solvolysate was then neutralized with 2 *M* sodium hydroxide and evaporated to dryness. The residue, dissolved in 0.5 *M* sodium hydroxide was percolated through a column of an Amberlite XAD-2 resin for desalting. After washing with water, the bile acids were eluted with ethanol-containing ammonia as described above. After evaporation of the solvent, the residue was hydrolysed, and the free bile acids were extracted as described for the unconjugated bile acid fraction.

The sulphated bile acids were treated as described for the taurine-conjugated bile acid fraction.

Capillary column gas chromatography

Before capillary GC, bile acids were converted into methyl ester dimethylethylsilyl ether (Me-DMES) derivatives with diazomethane and with 50 μl of dimethylethylsilylimidazole, as reported previously [10]. The solution of the bile acid derivatives was dissolved in hexane, washed with water and evaporated to dryness. An aliquot of the bile acid derivatives that were dissolved in 50 μl of hexane was injected into a capillary column gas chromatograph.

The gas chromatograph (Model GC-4BPF, Shimadzu) used was equipped with a flame-ionization detector. A Hicap CBP-1 capillary column (25 m×0.25 mm I.D., Shimadzu) was used at 290°C with nitrogen as the carrier gas.

Gas chromatography–mass spectrometry

The gas chromatograph–mass spectrometer used, a JMS D-300 (Japan Electron Optics Laboratory, Tokyo, Japan), had a 1% OV-1 (1 m×2 mm I.D.) column maintained at 250°C.

Thin-layer chromatography (TLC)

A TLC plate [16], 250 mm in thickness, was prepared using Kieselgel G (Merck). The solvent systems used were: system A, ethyl acetate–*n*-butanol–acetic acid–water (8:6:3:3, v/v); system B, benzene–acetic acid (90:10, v/v). Bile acids were detected by spraying with 5% phosphomolybdic acid in 5% acetic acid solution and heating for 5 min at 110°C.

RESULTS AND DISCUSSION

Glyco-$\alpha\alpha$-Na, tauro-$\beta\beta$-Na and glyco-$\alpha\alpha$ 7-sulphate-Na were newly synthesized for use as internal standards for quantitative GC analysis of urinary bile acids after group separation.

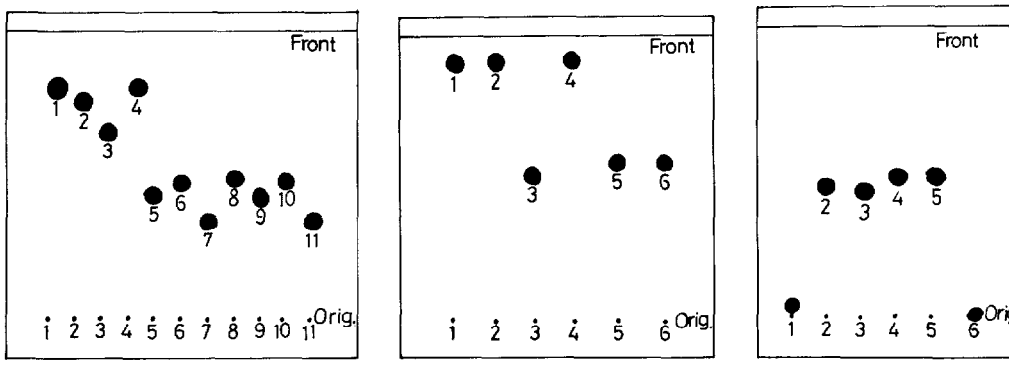

Fig. 2. Thin-layer chromatogram of the synthesized compounds and the natural bile acids. Solvent system A: ethyl acetate–*n*-butanol–acetic acid–water (8:6:3:3, v/v). Spots: 1 = glyco lithocholic acid; 2 = glyco chenodeoxycholic acid; 3 = glyco cholic acid; 4 = glyco-$\alpha\alpha$; 5 = glyco-$\alpha\alpha$ 7-sulphate; 6 = glyco lithocholic acid 3α-sulphate; 7 = glyco cholic acid 3α-sulphate; 8 = tauro lithocholic acid; 9 = tauro chenodeoxycholic acid; 10 = tauro-$\beta\beta$; 11 = tauro cholic acid.

Fig. 3. Thin-layer chromatogram of the solvolysates of synthesized compounds. Solvent system as in Fig. 2. Spots: 1 = glyco-$\alpha\alpha$; 2 = the solvolysate of glyco-$\alpha\alpha$ 7-sulphate (200 μg); 3 = glyco-$\alpha\alpha$ 7-sulphate; 4 = the solvolysate of glyco-$\alpha\alpha$; 5 = the solvolysate of tauro-$\beta\beta$; 6 = tauro-$\beta\beta$.

Fig. 4. Thin-layer chromatogram of the hydrolysates of glyco-$\alpha\alpha$ and tauro-$\beta\beta$. Solvent system B: benzene–acetic acid (90:10, v/v). Spots: 1 = glyco-$\alpha\alpha$; 2 = the hydrolysate of glyco-$\alpha\alpha$; 3 = $\alpha\alpha$; 4 = $\beta\beta$; 5 = the hydrolysate of tauro-$\beta\beta$; 6 = tauro-$\beta\beta$.

The TLC analysis shows each spot for the synthesized compounds and natural bile acids (Fig. 2). Each spot for the synthesized compounds was near the spots of conjugated lithocholic acids with glycine, taurine or sulphuric acid. In separate experiments, 30 and 200 μg of glyco-$\alpha\alpha$ 7-sulphate-Na were solvolysed. On the TLC plate, the behaviour of the product agreed with that of glyco-$\alpha\alpha$-Na, and no unchanged glyco-$\alpha\alpha$ 7-sulphate was detected in either case (Fig. 3). Besides, glyco-$\alpha\alpha$ and tauro-$\beta\beta$ were not converted into other compounds by solvolysis. Moreover, each 200-μg amount of glyco-$\alpha\alpha$-Na and tauro-$\beta\beta$-Na was completely converted by hydrolysis into $\alpha\alpha$ and $\beta\beta$, respectively, as shown in Fig. 4. These results seem to support the view that the recoveries in hydrolysis and solvolysis are the same for the synthesized bile acids as for natural conjugated bile acids.

When an internal standard solution, composed of $\beta\beta$, glyco-$\alpha\alpha$-Na, tauro-$\beta\beta$-Na and glyco-$\alpha\alpha$ 7-sulphate-Na, was analysed, $\beta\beta$ detected by GC of the unconjugated fraction and $\alpha\alpha$ by GC of the glycine-conjugated fraction were derived from glyco-$\alpha\alpha$-Na, $\beta\beta$ in the taurine-conjugated fraction from tauro-$\beta\beta$-Na and $\alpha\alpha$ in the sulphated fraction from glyco-$\alpha\alpha$ 7-sulphate-Na. Both peaks of $\alpha\alpha$ and $\beta\beta$ were not detected simultaneously in all of the four fractions. No peaks due to artifacts produced from internal standards during the analysis procedure were detected. These results indicate that the synthesized compounds, together with $\beta\beta$, are useful internal markers for the identification of bile acids by relative retention times and for group separation analysis of bile acids by PHP gel column chromatography.

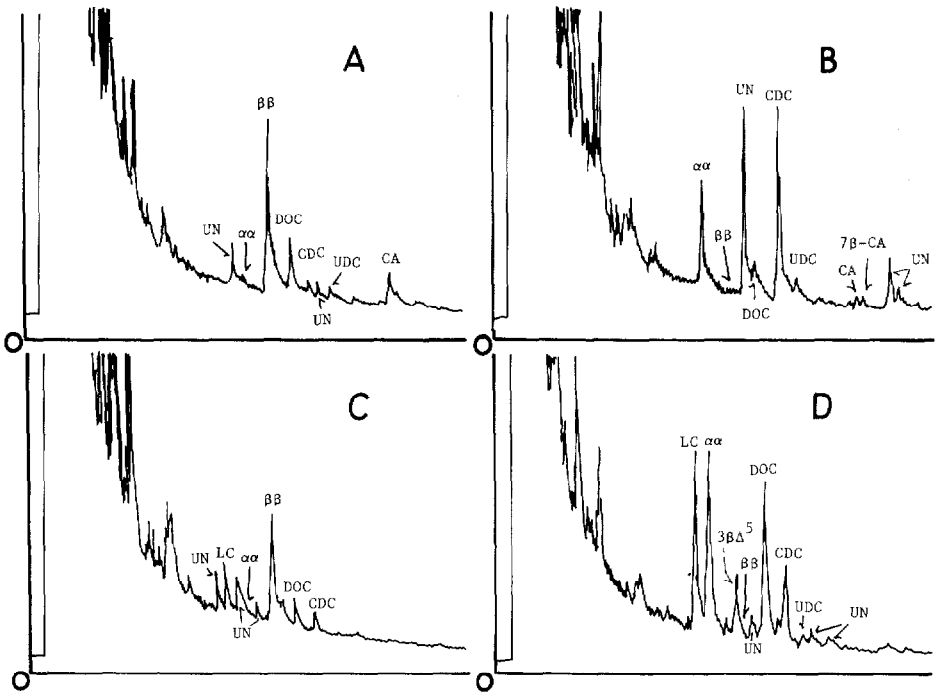

Fig. 5. Capillary column gas chromatograms of Me-DMES derivatives of bile acids after group separation of urinary bile acids by PHP gel column chromatography. (A) Unconjugated fraction; (B) glycine-conjugated fraction; (C) taurine-conjugated fraction; (D) sulphated fraction. Peaks: LC = lithocholic acid; DOC = deoxycholic acid; CDC = chenodeoxycholic acid; UDC = ursodeoxycholic acid; CA = cholic acid; 7β-CA = 3α,7β,12α-trihydroxycholanoic acid; $3\beta\Delta^5$ = 3β-hydroxy-5-cholenoic acid; UN = unidentified bile acid.

The four internal standards were used in the analysis of urinary bile acids. As expected, the internal standard peak appeared on each gas chromatogram: $\beta\beta$ in the free fraction (Fig. 5A), $\alpha\alpha$ in the glycine-conjugated fraction (Fig. 5B), $\beta\beta$ in the taurine-conjugated fraction (Fig. 5C) and $\alpha\alpha$ in the sulphated fraction (Fig. 5D). The mass spectra of these peaks agreed with those of the Me-DMES derivatives of authentic $\alpha\alpha$ and $\beta\beta$, respectively [10]. From many data obtained during our preliminary studies, no urinary substance had the same retention time as $\alpha\alpha$ or $\beta\beta$ in urine from many patients with liver disease. The internal standard peak was separated from the peak of urinary bile acids in all fractions analyzed by GC.

Furthermore, the use of glycine-conjugated, taurine-conjugated and sulphated internal standards can take into account the extent of solvolysis and hydrolysis in each fraction, as was also reported by Ghoos et al. [11]. The relationship between the bile acid concentration and the ratios of each bile to $\alpha\alpha$ [11] and to $\beta\beta$ [9], respectively, was found to be linear. These findings indicate that the absolute amount of individual bile acids in each fraction after group separation of urinary bile acids is determined.

The most reliable internal standard for accurate quantitation would be an isotopically labelled derivative of the bile acids to be determined, using gas chromatography–mass spectrometry. However, this is too difficult and complicated to achieve in multicomponent analyses of urinary bile acids.

Therefore, the newly synthesized compounds, together with $\beta\beta$, which can be added to a sample prior to extraction, are reliable internal standards for quantitative GC of bile acids after group separation of urinary bile acids.

REFERENCES

1 M.T.R. Subbiah, J. Lipid Res., 14 (1973) 692.
2 C.B. Cambell, C. McGuffie and L.W. Powell, Clin. Chim. Acta, 63 (1975) 249.
3 T. Laatikainen and A. Hesso, Clin. Chim. Acta, 64 (1975) 63.
4 G. Deleze, G. Karlaganis, W. Giger, M. Reinhard, D. Sibiropoulos and G. Paumgartner, in G. Paumgartner and A. Stiehl (Editors), Proceedings of the 4th Bile Acid Meeting, Basle, Oct. 11–12, 1976, MTP Press, Lancaster, 1976, pp. 59–62.
5 P.E. Ross, C.R. Pennington and I.D.A. Bouchier, Anal. Biochem., 80 (1977) 458.
6 B. Alme, A. Bremmelgaard, J. Sjovall and P. Thomassen, J. Lipid Res., 18 (1977) 339.
7 T. Laatikainen, P. Lehtonen and A. Hesso, Clin. Chim. Acta, 85 (1978) 145.
8 G. Karlaganis and G. Paumgartner, J. Lipid Res., 19 (1978) 771.
9 P. Back and K. Walter, Gastroenterology, 78 (1980) 671.
10 K. Arimoto, K. Adachi and N. Yamaga, Steroids, 39 (1982) 631.
11 Y. Ghoos, P. Rutgeerts and G. Vantrappen, J. Lipid Res., 24 (1983) 1376.
12 K. Tserng, D.L. Hachey and P.D. Klein, J. Lipid Res., 18 (1977) 404.
13 J. Goto, H. Kato, F. Hasegawa and T. Nambara, Chem. Pharm. Bull., 27 (1979) 1402.
14 J. Roovers, E. Evrard and H. Vanderhaeghe, Clin. Chim. Acta, 19 (1968) 449.
15 J. Goto, M. Hasegawa, H. Kato and T. Nambara, Clin. Chim. Acta, 87 (1978) 141.
16 G. Parmentier and H. Eyssen, J. Chromatogr., 152 (1978) 285.